Bacterial Polysaccharides

II. Laboratory-scale Production of Polysaccharides by Species of *Xanthomonas*[1,2]

V. G. Lilly, H. A. Wilson, and J. G. Leach

Department of Plant Pathology, Bacteriology and Entomology, West Virginia University, Morgantown, West Virginia

Received for publication August 19, 1957

The most intensively studied bacterial polysaccharide is the dextran produced by *Leuconostoc mesenteroides* and related species. Most of the exocellular bacterial polysaccharides which have been studied have been produced on agar media. Of the bacterial polysaccharides produced by plant pathogenic bacteria, the glucosan produced by *Agrobacterium tumefaciens* appears to have been the most thoroughly investigated (Putman *et al.*, 1950). Cooper and Preston (1935) reported a number of plant pathogenic bacteria which synthesize polysaccharides. Most of the investigators who have worked with the polysaccharides (exudates, gums, and so forth) produced by plant pathogenic bacteria have been interested in the biological properties of these substances rather than techniques of production.

Species of the genus *Xanthomonas* are plant pathogens which produce exudates at the site of lesions on infected plants. This work had for its purpose a study of the environmental and nutritional factors which affect the production of polysaccharide by *Xanthomonas phaseoli*, and certain other species of this genus.

Materials and Methods

Organisms. The species of *Xanthomonas* used were: *Xanthomonas phaseoli* (USDA 2272); *X. campestris* (Dr. G. E. Pound, University of Wisconsin); *X. malvacearum* (ATCC 9924); *X. translucens* f. sp. *hordeiavenae*, (ATCC 9000); *X. translucens* f. sp. *undulosa*, (ATCC 9002); *X. carotae* (ATCC 10,547); *X. hederae*, (ATCC 9653); *X. papavericola*, (ATCC 10,204); and *X. vesicatoria*, (isolated in our laboratories).

Basal medium. Modifications of the following basal medium were used. Glucose (anhydrous Cerelose[3]), 25 g; enzymatic casein hydrolysate,[4] 2 g; fumaric acid, 2 g; KH_2PO_4, 1 g; Na_2CO_3, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe (III) and Zn (II), 0.2 mg each; Mn (II), 0.1 mg; distilled water, 1 L. The microelements were added as 2 ml of a master solution which contained per L: $Fe(NO_3)_3 \cdot 9H_2O$, 723 mg; $ZnSO_4 \cdot 7H_2O$, 439.8 mg; and $MnSO_4 \cdot 4H_2O$, 203.0 mg (Lilly and Barnett, 1951). The pH was adjusted to 6.0 with NaOH before autoclaving.

Fermentation vessels. The bacteria were grown in 9-L Pyrex serum bottles no. 670[5] under aeration. The aeration assembly and air filter are shown in figure 1. The necks of the serum bottles were wrapped with sufficient cotton and covered with gauze tape so that the aeration assembly fitted snugly. The medium (3 L) was introduced into the bottles before the aerator assembly and air filter were lashed in place. The entire unit was autoclaved (121 C) for 1 hr, shorter times of heating were insufficient to ensure sterility.

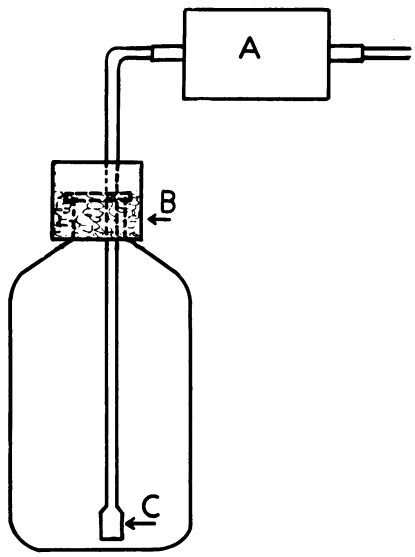

Figure 1. Fermentation vessel. *A*. Cotton-filled air filter. *B*. Aerator head made from a tin can. The sections of the air line passing through the heads of the air filter and the aerator assembly were 3 in. lengths of threaded ⅛ in. pipe. *C*. Fritted glass gas dispenser tube.

Aeration. A pressure regulator was introduced into the compressed air line ahead of the manifold, and the air pressure was measured with an open end mercury manometer. The air pressure used varied (5 to 18 cm Hg) depending on the amount of foaming encountered. The sterile air was dispensed in the medium by means of a fritted-glass gas dispersion tube no. 39533, 12c.[5] An air flow of about 5 L per min was obtained with the

[1] Published with the approval of the Director of West Virginia Agricultural Experiment Station as Scientific Paper No. 559.

[2] The first paper in this series was published in Phytopathology, 1957, **47**, 113–120.

[3] Corn Products Refining Co., Argo, Illinois.

[4] Nutritional Biochemical Corp., Cleveland, Ohio.

[5] Corning Glass Works, Corning, New York.

pressure at 15 cm Hg. Aeration appeared to be fairly uniform at the beginning of an experiment, but rapidly decreased as the viscosity of the medium increased.

Inoculum. The inoculum was grown in 50 ml of nutrient broth in 250-ml Erlenmeyer flasks. The cultures were placed on a Brunswick rotary shaker[6] and incubated at 25 C for 2 to 3 days before use. The inoculum flasks were stoppered with cotton and capped with 50-ml beakers. Fifty ml of inoculum was added to each bottle.

Polysaccharide determination. The polysaccharides were precipitated by the addition of 3 volumes of acetone to the culture media. The precipitate was carefully collected on an L-shaped glass rod and repeatedly worked with fresh acetone. Omission of this step resulted in a dark intractable material after drying. As much acetone as possible was removed by pressing, the remainder being removed under vacuum. The polysaccharides were dried overnight at 55 C before being weighed. Most of the polysaccharides were light-colored fluffy materials, provided the precipitation and washing with acetone were conducted properly. The acetone extracted most of the yellow pigment synthesized by these species. Ethyl alcohol was also satisfactory as a precipitating and dehydrating agent.

Results

The results obtained with *Xanthomonas phaseoli* during the preliminary stages of the investigation are summarized below. Little or no polysaccharide was formed unless the cultures were aerated. Within limits, the yield of polysaccharide was proportional to the amount of aeration; increasing the pressure from 5 to 15 cm Hg increased the yield of polysaccharide from 3 to 9 g per L. Foaming was a serious problem until a suitable antifoam agent was found.

Polysaccharide was produced in a temperature range of 25 to 35 C, the highest yield being obtained at 33 C. No polysaccharide was produced at 40 C. The polysaccharide produced at 33 and 35 C was difficult to free from dark-colored material which was precipitated by acetone. The polysaccharide produced at 25 and 28 C was a light colored fluffy material. Temperatures below 25 C were not investigated. The remainder of the experiments with *X. phaseoli* and the other species tested were made at 28 C.

Polysaccharide formation was rapid. The yields of acetone-precipitated material after 1, 2, 3, 4, and 6 days of incubation were 1.7, 5.0, 7.4, 7.8, and 9.7 g per L, respectively. A 5-day period of incubation was used in other experiments with *X. phaseoli*.

The following modifications of the basal medium were tried. Media which contained 1, 2 and 4 g per L of enzymatic casein hydrolysate yielded 7.1, 8.6, and 10.1 g of acetone-precipitated polysaccharide, respectively. Acid-hydrolyzed casein was as satisfactory as enzymatic casein hydrolysate. Soy bean alpha protein[4] was unsatisfactory; excess foaming was encountered despite the use of an antifoam agent. Omission of all the constituents of the medium except glucose and enzymatic casein hydrolysate reduced the yield to about 2 g per L.

Glucose concentration. The yield of polysaccharide was determined when the glucose content of the medium was varied. No antifoam agent was used in these runs. The data are given in table 1. It will be noted that the best percentage yield was obtained when the medium contained 10 g of glucose per L; the largest yields per L being obtained when 50 g of glucose were used per L.

Carbon sources. Preliminary data on the production of polysaccharide from various carbon sources by *X. phaseoli* have been published (Leach *et al.*, 1957). Because of the large scale of the experiments, particular attention was devoted to those carbon sources which are abundant in supply and reasonable in cost. All carbon sources were used at a rate which supplied 10 g C per L (table 2.)

In addition, lactose, galactose, raffinose, and cellulose were investigated as carbon sources, but polysaccharide production on them was poor.

Antifoam agents. Excessive foaming, which occurred

TABLE 1

*The effect of glucose concentration on the yield of polysaccharide by Xanthomonas phaseoli**

Glucose	Polysaccharide	Percentage Yield Based on Glucose
g/L	*g/L*	
5	3.3	64
10	7.9	79
20	8.4	42
25	9.0	36
50	11.2	22

* Incubation 5 days at 28 C. No antifoam used.

TABLE 2

*Yield of acetone precipitated polysaccharide produced by Xanthomonas phaseoli on various carbon sources**

Carbohydrate	Yield
10 g C/L	*g/L*
Glucose	9.4
Maltose	6.2
Sucrose	8.0
Soluble starch	6.8
Corn starch	8.9
Corn starch hydrolyzed with Diamalt II	10.1†

* Incubation 5 days at 28 C. No antifoam used; air pressure 14 to 18 cm Hg.

† The starch was boiled, cooled and diluted. Dry Fleishman Diamalt (Standard Brands, Inc., New York, New York), 27 g per 100 g starch was added. The solution was held at 37 C for 24 hr before incorporating the digest in the medium.

[6] New Brunswick Scientific Co., New Brunswick, New Jersey.

in many of the early experiments, caused loss of material, contamination, and inconvenience. Suitable antifoam agents were sought. Corn oil reduced yields and was not effective in preventing foaming. Silicone[7] antifoam A reduced foaming satisfactorily, but also reduced yields. A series of Hodag antifoam agents[8] were tested. Some of these results are presented in table 3.

The antifoam agents were added in the amounts indicated in table 3 and autoclaved with the basal medium. Presumably, more efficient use of these agents and better yields would have been obtained had these materials been added as needed during fermentation.

Polysaccharide production by other Xanthomonas species. The basal medium was modified by adding 0.5 ml of an antifoam agent (Hodag KP-2). Six fermentation bottles were used for each organism. The polysaccharide was harvested at the end of 5 days when the organisms were growing rapidly. The run was divided for some slow-growing species, three bottles being harvested at 5 days, the others later. The essential data are presented in table 4. The polysaccharide produced by most species had a fibrous appearance after precipitation with acetone. The polysaccharide produced by *X. vesicatoria* was exceptionally difficult to precipitate and to free from a dark-colored gum.

Additional experiments were made with *Xanthomonas hederae*. By increasing the time of incubation to 10 days, a yield of 5.6 g per L of polysaccharide was obtained. Sucrose was less suitable than glucose for polysaccharide formation by this organism (3.7 g per L at 9 days).

[7] Dow-Corning Corp., Midland, Michigan.
[8] Hodag Chemical Co., Chicago, Illinois.

TABLE 3

*The effect of various antifoam agents on the production of polysaccharide by Xanthomonas phaseoli**

Antifoam Agent	Amount Used	Yield	Percentage Yield Based on Glucose
	ml/L	*g/L*	
Hodag KF	3	6.4	25.6
Hodag K6	3	5.3	21.2
Hodag KS	3	3.7	14.8
Hodag KP-1	3	4.7	18.8
	1	9.6	38.2
Hodag KP-2	3	9.0	36.0
	1	11.0	44.0
	0.5	15.0	60.0

* Incubation 5 days at 28 C; air pressure 15 cm Hg.

TABLE 4

*Yields of polysaccharide by various species and subspecies of the genus Xanthomonas**

Organism	Yield	Percentage Yield Based on Glucose
	g/L	
X. campestris	9.9	39.6
X. malvacearum	9.6	38.3
X. translucens f. sp. *hordei-avenae*	5.1	20.4
X. translucens f. sp. *undulosa*	2.2	8.8
X. carotae	6.5	26.0
X. hederae	2.4	9.6
X. hederae (7 days)	3.4	13.6
X. papavericola	1.8	7.2
X. papavericola (8 days)	3.3	13.2
X. vesicatoria	1.9	7.6

* Incubation 5 days (except where noted) at 28 C; air pressure 15 cm Hg.

Discussion

Xanthomonas phaseoli differs from *Leuconostoc mesenteroides* in that it produces polysaccharide from glucose, and carbohydrates which yield glucose on hydrolysis. In this, *X. phaseoli* resembles a number of sporeforming bacteria studied by Forsyth and Webley (1949). The preliminary evidence (Hedrick, 1956) indicates that *Xanthomonas* species synthesize heteropolysaccharides or complex mixtures of homopolysaccharides. Additional studies have shown that at least six monosaccharides are present in hydrolysates of *X. phaseoli* polysaccharide (Unpublished). It is probable that polysaccharide formation by *Xanthomonas* species is a more complex process than the formation of homopolysaccharides by *L. mesenteroides* and *Agrobacterium tumefaciens*.

Of the species tested, *Xanthomonas phaseoli*, *X. campestris*, and *X. malvacearum* appear to be the most suitable for the production of polysaccharides. All three species produced approximately 10 g of polysaccharide per L when the medium contained 25 g of glucose per L. It is probable that this yield could be doubled if adequate aeration and agitation were used. Thus, when only 10 g per L of glucose was used *X. phaseoli* converted about 80 per cent of the glucose into polysaccharide.

Foaming is a serious problem unless suitable antifoam agents are used. Of those tested, Hodag KP-2 appeared to be the best. It is possible that other antifoam agents would be superior. It is believed that the addition of antifoam agents as needed during fermentation would be advantageous.

It is thought that the role of the polysaccharides produced by plant pathogenic bacteria is to protect these species from desiccation and other adverse conditions (Leach *et al.*, 1957). We have used these polysaccharides with some success for the preservation of another species of bacteria in the dry state. On the basis of preliminary tests, the polysaccharides produced by species of *Xanthomonas* are poor carbon sources for bacteria and fungi. For this reason, these substances might be of value as agents which promote or stabilize soil aggregation. In this connection, it may be noted

that most of the constituent sugars found by Whistler and Kirby (1956) in polysaccharides isolated from soil are also found in the polysaccharides produced by various species of *Xanthomonas*.

ACKNOWLEDGMENTS

This work was supported in part by a contract with the Chemical Corps, Fort Detrick, Frederick, Maryland. We wish to thank the Hodag Chemical Co. for the gift of various antifoam agents.

SUMMARY

A suitable medium for the production of polysaccharides by *Xanthomonas* species consists of glucose (starch or sucrose), enzymatic casein hydrolysate, and salts. Adequate aeration and agitation are necessary, as well as a foam suppressing agent (Hodag KP-2 is suitable). The polysaccharides may be precipitated with acetone or ethanol. The following species produced 6 or more g of polysaccharide per L: *Xanthomonas phaseoli*, *X. campestris*, *X. malvacearum* and *X. carotae*. The other species tested (*X. translucens* f. sp. *hordei-avenae* and f. sp. *undulosa*, *X. hederae*, *X. papavericola*, and *X. vesticatoria*) were less productive.

REFERENCES

COOPER, E. A. AND PRESTON, J. F. 1935 Enzyme formation and polysaccharide synthesis by bacteria. Biochem. J., **29**, 2267–2277.

FORSYTH, W. G. C. AND WEBLEY, D. M. 1949 Polysaccharides synthesized by aerobic mesophilic spore-forming bacteria. Biochem. J., **44**, 455–459.

HEDRICK, H. G. 1956 Exudates produced by phytopathogenic bacteria (abstract). Phytopathology, **46**, 14–15.

LEACH, J. G., LILLY, V. G., WILSON, H. A., AND PURVIS, M. R., JR. 1957 Bacterial polysaccharides: The nature and function of the exudate produced by *Xanthomonas phaseoli*. Phytopathology, **47**, 113–120.

LILLY, V. G. AND BARNETT, H. L. 1951 *Physiology of the fungi*, p. 421. McGraw-Hill Book Co., Inc., New York.

PUTMAN, E. W., POTTER, A. L., HODGSON, R., AND HASSID, W. Z. 1950 The structure of crown-gall polysaccharide. I. J. Am. Chem. Soc., **72**, 5024–5026.

WHISTLER, R. L. AND KIRBY, K. W. 1956 Composition and behavior of soil polysaccharides. J. Am. Chem. Soc., **78**, 1755–1759.

Cellulose Decomposition in Soil Burial Beds

I. Soil Properties in Relation to Cellulose Degradation[1]

E. L. SCHMIDT AND O. R. RUSCHMEYER[2]

Department of Bacteriology and Immunology and Department of Soils, University of Minnesota, St. Paul, Minnesota

Received for publication August 26, 1957

Various modifications of a soil burial test came into widespread use during World War II to test the effectiveness of chemical treatments in the prevention of microbiological deterioration of cellulosic materials. In soil burial tests, fabric strips treated with the test preservative are buried and exposed to the activity of the cellulolytic microflora of a composted soil bed. The change in tensile strength of the sample during incubation is taken as a measure of the effectiveness of the preservative treatment. Exposure to the varied cellulolytic microflora in a composted soil is considered the most rigorous and practical means for the evaluation of antideterioration treatments of fabrics. Of much concern is the possibility that soil burial tests conducted in different laboratories using different soil mixtures may provide erratic results in the evaluation of the same treatment (Siu, 1951).

The utility of the soil burial test in relation to other fabric testing procedures has been discussed by Bertolet (1944), Marsh *et al.* (1945), and by Siu (1951). Marsh *et al.* (1945) assumed that differences in soils with respect to physical, chemical, and biological properties might contribute to considerable variability in soil burial test data. In tests with four different soils, they experienced some variation but stated that results were more consistent than had been expected.

Other comparative soil burial tests were carried out in 1944 under a program directed by the American Association of Textile Chemists and Colorists (1945). Replicate sets of treated fabric samples were tested in soil burial by 12 cooperating laboratories in industry and government, using broadly standardized procedures outlined for the testing program. Good agreement

[1] Paper No. 3637 of the Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Associate Professor in the Department of Bacteriology and Immunology and in the Department of Soils, and Research Assistant in the Department of Bacteriology and Immunology, respectively, University of Minnesota.